review article

TGF-β signalling from cell membrane to nucleus through SMAD proteins

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The recent identification of the SMAD family of signal transducer proteins has unravelled the mechanisms by which transforming growth factor-β (TGF-β) signals from the cell membrane to the nucleus. Pathway-restricted SMADs are phosphorylated by specific cell-surface receptors that have serine/threonine kinase activity, then they oligomerize with the common mediator Smad4 and translocate to the nucleus where they direct transcription to effect the cell's response to TGF-β. Inhibitory SMADs have been identified that block the activation of these pathway-restricted SMADs.

TGF-β1 is the prototype of a large family of cytokines that includes the TGF-βs, activins, inhibins, bone morphogenetic proteins (BMPs) and Müllerian-inhibiting substance (reviewed in ref. 1) (Table 1). Members of the TGF-β family exert a wide range of biological effects on a large variety of cell types, for example they regulate cell growth, differentiation, matrix production and apoptosis. Many of them have important functions during embryonal development in pattern formation and tissue specification; in the adult they are involved in processes such as tissue repair and modulation of the immune system.

Here we discuss recent breakthroughs in our understanding of the mechanisms used by members of the TGF-β family to elicit their effects on target cells, focusing on the pivotal role of SMAD proteins in relaying signals from cell-surface receptors to the nucleus.

Signalling through receptor complexes

TGF-β family members initiate their cellular action by binding to receptors with intrinsic serine/threonine kinase activity. This receptor family consists of two subfamilies, type I and type II receptors, which are structurally similar, with small cysteine-rich extracellular regions and intracellular parts consisting mainly of the kinase domains. Type I receptors, but not type II receptors, have a region rich in glycine and serine residues (GS domain) in the juxtamembrane domain. Each member of the TGF-β superfamily binds to a characteristic combination of type I and type II receptors (Table 1), both of which are needed for signalling.

Studies of the receptors for TGF-β have provided a model for the activation of these serine/threonine kinase receptor complexes[2]. TGF-β1 first binds to the type II receptor (TβR-II), which occurs in the cell membrane in an oligomeric form with activated kinase[3,4]. Then, the TGF-β type I receptor (TβR-I), which may also occur in an oligomeric form[5] and cannot bind TGF-β in the absence of TβR-II, is recruited into the complex; TβR-II phosphorylates TβR-I in the GS domain to activate it. The assembly of the receptor complex is triggered by ligand binding, but the complex is also stabilized by direct interaction between the cytoplasmic parts of the receptors[6]. The model[2] predicts that the type II and type I receptors act in sequence, which is supported by the finding that a constitutively active TβR-I (Thr 204 replaced with an aspartate residue) is able to exert TGF-β signals in the absence of TβR-II (ref. 7). It is likely that other serine/threonine kinase receptor complexes are also activated by a similar mechanism[8,9], although some variations on the theme have been noted. One of the TGF-β isoforms (TGF-β2) binds only with low affinity to TβR-II and requires the cooperation with TβR-I or betaglycan, an accessory transmembrane proteoglycan, for high-affinity binding[10]. Moreover, BMPs bind

Table 1 TGF-β family members, their receptors and signalling molecules

Subfamily	TGF-β	Activin	BMP
Examples of ligands	TGF-β1 TGF-β2 TGF-β3	Activin A	BMP-2 BMP-4 BMP-7/OP-1
Type II receptors	TβR-II	ActR-II ActR-IIB	BMPR-II ActR-II ActR-IIB
Type I receptors	TβR-I	ActR-I? ActR-IB	BMPR-IA BMPR-IB ActR-I
Pathway-restricted SMADs	Smad2 Smad3	Smad2 Smad3	Smad1 Smad5 Smad9?
Common-partner SMAD	Smad4	Smad4	Smad4
Inhibitory SMADs	Smad6 Smad7	Smad6 Smad7	Smad6 Smad7
Responses	Inhibition of mitogenicity Induction of extracellular matrix	Induction of dorsal mesoderm Induction of erythroid differentiation Induction of follicle-stimulating hormone release	Induction of ventral mesoderm Induction of cartilage and bone Induction of apoptosis

The three best-characterized vertebrate TGF-β subfamiles are listed.

with low affinity to BMP type I or type II receptors individually, and with high affinity only when the two BMP receptor types are presented together[11–13].

Analysis of ^{125}I-labelled TGF-β1 crosslinked to its receptors has suggested that the signalling complex is a heterotetramer consisting of two TβR-I and two TβR-II molecules[14]. This conclusion is supported by experiments using chimaeric erythropoietin/TGF-β receptors which showed that both homodimerization of type I receptors and hetero-oligomerization with the type II receptor are needed for the antimitogenic effect[5]. Moreover, studies of a series of signalling-defective TβR-I receptors revealed that a kinase-defective TβR-I can complement an activation-defective TβR-I, suggesting that the signalling complex consists of at least two TβR-I molecules[15].

In the receptor-activation model, TβR-I acts downstream of TβR-II for most, if not all, TGF-β-mediated responses, and the type I receptor thus determines the specificity of the intracellular signals[16]. A nine-amino-acid sequence between kinase subdomains IV and V of TβR-I, which diverges between the different type I receptors, is important for transduction of specific TGF-β signals[17] (Fig. 1).

Situations have been described where, as a result of a decrease in expression of TβR-II but not TβR-I, cells lose the antiproliferative response to TGF-β, whereas the matrix accumulation induced by TGF-β is retained[18,19]. Moreover, expression of a dominant-negative TβR-II was found to block the growth-inhibitory effect of TGF-β, but not the effect on extracellular matrix[19,20]. These observations are compatible with a more important role for TβR-II in the antiproliferative response than in the matrix response, but they do not necessarily contradict the sequential activation model in which activated TβR-I is required for both responses; different effects of TGF-β may occur at different threshold levels of stimulation, with the antiproliferative effect requiring a more efficient stimulus than the effect on matrix, for example.

An important step in receptor activation is phosphorylation of the tetrameric receptor complex. Phosphorylation sites in TβR-II and TβR-I have been mapped using wild-type and chimaeric receptors[21–23] (Fig. 1). Certain of the phosphorylation sites in TβR-II are important in modulating the signalling activity of the receptor; phosphorylation of Ser 213 and Ser 409 is required for TβR-II activity, whereas phosphorylation of Ser 416 inhibits TβR-II signalling[22]. Notably, TβR-II and the activin type IIB receptor autophosphorylate on tyrosine residues, as well as on serine and threonine residues, and so may function as dual-specificity kinases[23,24]. The importance of autophosphorylation on tyrosine residues remains to be determined.

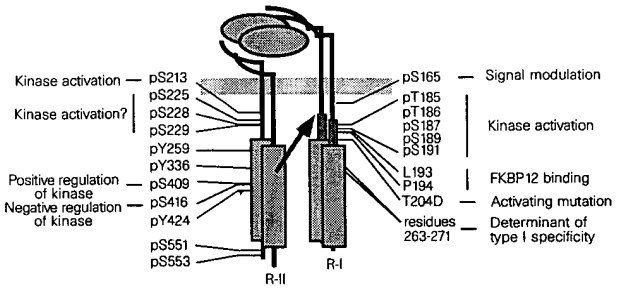

Figure 1 An activated TGF-β-receptor complex. The dimeric TGF-β molecule (light blue) binds to a heterotetrameric complex of two TβR-I and two TβR-II molecules. The GS domain (red) just upstream of the kinase domain (dark blue) of TβR-I is indicated. Known autophosphorylation sites in TβR-II and sites in TβR-I phosphorylated by TβR-II and their functional roles are indicated, as well as amino-acid residues involved in FKBP12 binding, activating mutation (T204D) and determination of TβR-I specificity in signalling (residues 263-272). Assignments of phosphorylation sites are based on data in refs 21-23. The amino-acid numbers for sites in the TβR-II in ref. 21 differ by two owing to a mistake in the amino-acid numbering in this reference.

TβR-I is phosphorylated by TβR-II at several residues in the GS domain, which leads to activation of the TβR-I kinase[2,21] (Fig. 1). It is possible that phosphorylation of TβR-II in the corresponding part of the juxtamembrane region[22] is also important for its activation. In addition, TβR-I is phosphorylated at Ser 165, which is located N-terminally of the GS domain. Mutation of Ser 165 gave a type I receptor with a more powerful signalling effect in growth inhibition and matrix accumulation, but a weaker apoptotic signal[21]. Thus phosphorylation of Ser 165 may modulate TGF-β signalling.

Downstream signalling mechanisms

Recent studies in the genetically accessible *Drosophila* and *Caenorhabtidis elegans* have led to a breakthrough in our understanding of how signals are transduced from serine/threonine kinase receptors to the nucleus.

In *Drosophila*, the BMP-2/4 homologue Decapentaplegic (Dpp) acts by binding to the type II receptor Punt and to the type I receptors Thick veins and Saxophone. In a genetic screen for dominant enhancers of weak *dpp* alleles, *mothers against dpp* (*Mad*) and *Medea* were discovered[25,26]. Homozygous *Mad* mutants were found to have a phenotype similar to *dpp* mutants, with defects in midgut morphogenesis, imaginal disc development and embryonic dorsal–ventral patterning[26]. Evidence that Mad is a downstream component in the Dpp pathway came from the finding that *Mad* partially rescued the eye phenotype of dpp^{blk}[27], that Mad is required for the response to Dpp of the visceral mesoderm or endoderm[28], and that *Mad* mutations suppress dominant *thick veins* alleles[29]. There is also biochemical evidence that Mad functions downstream of Dpp receptors in *Drosophila*[30].

In *C. elegans*, *daf-1* and *daf-4* encode serine/threonine kinase receptors. *Daf-4* mutants are dauer-constitutive and smaller than wild-type; moreover, females are defective in egg-laying and males have fused tail rays. Screening for mutants with similar phenotypes revealed three genes, *sma-2*, *sma-3* and *sma-4*, which proved to be homologous to *Mad* of *Drosophila*[31]. As Sma-2 acts in the same cell as Daf-4 and *daf-4* is unable to rescue *sma-2* mutations, it was concluded that Sma molecules are involved in downstream signalling from the Daf-4 receptor.

SMADs are cytoplasmic mediators

At least nine genes homologous to *Mad* and *sma* have been identified in *Xenopus*, mouse and man, and shown to be components in signal transduction pathways downstream of serine/threonine kinase receptors (reviewed in ref. 1) (Fig. 2a). In an attempt to simplify the nomenclature, the designation *Smad* has been suggested for vertebrate homologues of *Sma* and *Mad*.

SMADs are molecules of relative molecular mass 42K–60K with two regions of homology at the amino and carboxy terminals, termed Mad-homology domains MH1 and MH2, respectively, which are connected with a proline-rich linker sequence (Fig. 2b). Recent work, which will be discussed below, suggests that in their inactive configurations, the MH1 and MH2 domains of SMADs make contact with each other: after activation by receptors, the molecules open up, form hetero-oligomeric complexes, and translocate to the nucleus where the transcription of target genes is affected.

Pathway-restricted SMADs. Different members of the SMAD family have different roles in signalling. Smad1, Smad2, Smad3 and possibly Smad5 interact with and become phosphorylated by specific type I serine/threonine kinase receptors and thereby act in a pathway-restricted fashion. An initial indication of a functional subspecialization among different SMADs came from the finding that *Xenopus* Smad1 (Xmad1) induces ventral mesoderm, a BMP

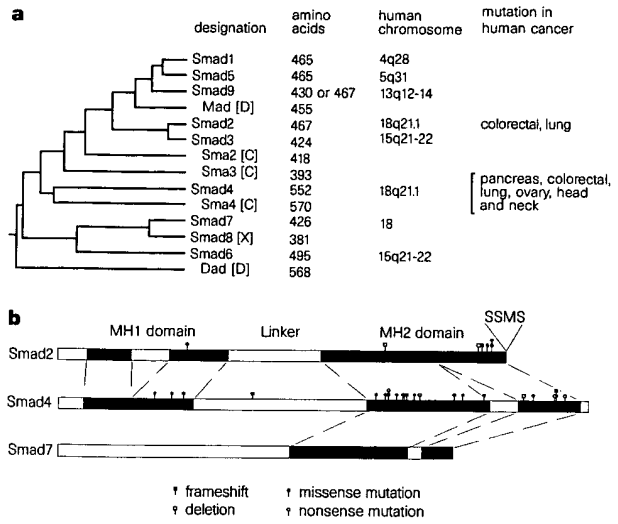

Figure 2 The SMAD family. **a**, A phylogenetic tree of human SMADs, Smad8 from *Xenopus* (X), Mad and Dad from *Drosophila* (D) and Sma from *C. elegans* (C) are shown. Alternative designations are as follows: Smad1 (Madr1, Xmad1, bsp1, Dwf-A, JV4-1), Smad2 (Madr2, Xmad2, JV18-1), Smad3 (hMad3, JV15-2), Smad4 (DPC4), Smad5 (Dwf-C, JV5-1), Smad6 (JV15-1) and Smad9 (MADH6). **b**, The sequences of representatives of pathway-restricted SMADs (Smad2), common-partner SMADs (Smad4) and inhibitory SMADs (Smad7) are shown to illustrate areas of homology between various types of SMAD molecules (black). Mutations in Smad2 and Smad4 detected in human cancers are indicated.

response[32,33], whereas Smad2 (Xmad2) induces dorsal mesoderm, an activin or Vg-1 response[32]. SMAD molecules are well conserved and act across species: human Smad1 (ref. 34), as well as *Drosophila* Mad[28], has ventralizing activity on *Xenopus* mesoderm, and mouse[35] and human[36] Smad2 have dorsalizing activity.

A picture is emerging in which different pathway-restricted SMADs couple to different receptors (Table 1). Smad2 and Smad3 are phosphorylated and translocated to the nucleus after stimulation by TGF-β[36–38] or activin (ref. 39 and A. Shimizu *et al.*, unpublished observation). Smad2 and Smad3 are very similar in their structures (Fig. 2a), and it is not surprising that there may be some redundancy in the functional activity between these two members of the family. Smad1 is phosphorylated and translocated into the nucleus after stimulation with BMP-2 (refs 29, 40) or BMP-4 (ref. 34). Smad5 induces ventral mesoderm in *Xenopus*[41], and the recently described Smad9/MADH6 (ref. 42) is structurally similar to Smad1 and Smad5; these molecules may also be involved in BMP signalling. There are reports that TGF-β also induces the phosphorylation of Smad1 (refs 43, 44), which may reflect redundancy or cooperativity in signalling (or a crossreactivity of antisera).

The phosphorylation of pathway-restricted SMADs by type I receptors triggers their activation. In the most C-terminal regions, pathway-restricted SMADs have a characteristic Ser-Ser-X-Ser (SSXS) motif, the two-most C-terminal serine residues of which are phosphorylated by type I receptors[40,45–47]. Pathway-restricted SMADs bind directly to type I receptors, as demonstrated by the co-immunoprecipitation of Smad2 or Smad3 with the type I and type II receptors affinity-crosslinked with ^{125}I-labelled TGF-β (refs 37, 38, 45). The association between TβR-I and Smad2 or Smad3 is dependent on the kinase activity of TβR-II, but was seen only with the kinase-inactive form of TβR-I and not with wild-type TβR-I (refs 38, 45). Moreover, the phosphopeptide maps of Smad1 phosphorylated in BMP-stimulated cells were similar to those of Smad1 phosphorylated *in vitro* by purified BMP type I receptor[40]. Taken together, these data suggest that the pathway-restricted SMADs are direct substrates of the type I receptor kinases, although a possible involvement of other kinases in SMAD activation has not been excluded. The data also suggest that the interaction between pathway-restricted SMADs and type I receptors is transient; presumably, SMADs are released from the receptors after phosphorylation. This idea is further supported by the observation that Smad2 molecules mutated at the three serine residues in the SSXS motif stably bind to the receptor and have dominant-negative effects[45–47].

Common-mediator SMADs. The mode of action of Smad4 differs from those of other members of the SMAD family. After ligand stimulation and phosphorylation of pathway-restricted SMADs, Smad4 forms hetero-oligomers with pathway-restricted SMADs[37,40,48,49], which in turn translocate into the nucleus and activate transcriptional responses (Fig. 3a). In mammalian cells, Smad4 forms complexes with Smad2 and Smad3 after activation of TGF-β or activin type I receptors[38,48,49], whereas it forms complexes with Smad1 (refs 40, 48), and possibly with Smad5 and Smad9, after activation of BMP type I receptors. Consequently, injection of Smad4 messenger mRNA into *Xenopus* animal caps induces both ventral and dorsal mesoderm[48,50] through the formation of complexes with Smad1, Smad5 or Smad9 and Smad2 or Smad3, respectively. Smad4, which lacks the C-terminal SSXS motif, does not bind to, nor is it phosphorylated by, TGF-β or BMP receptors[37,38,45,48]. The phosphorylation of Smad4 has been reported to increase after activin stimulation[48], although the functional importance of this remains to be determined.

So far, only Smad4 has been identified as a common-mediator SMAD in vertebrates. A Smad4 homologue has been identified in *Drosophila* (ref. 51; and P. Das and R. W. Padgett, personal com-

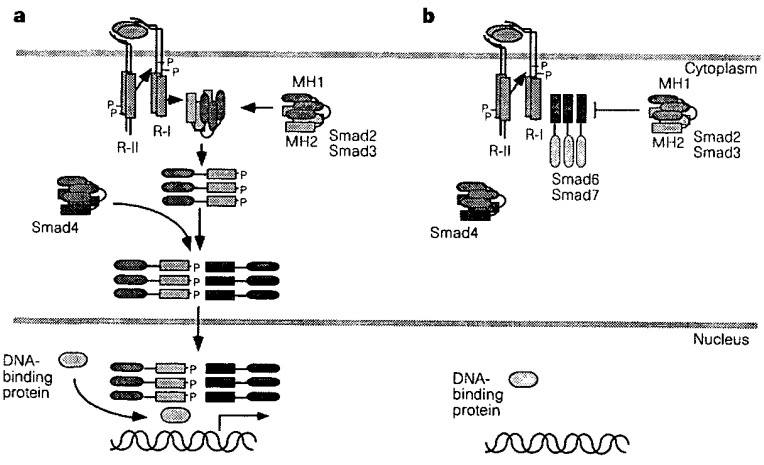

Figure 3 Agonistic and antagonistic SMAD proteins in TGF-β signalling. **a**, A hypothetical signal transduction pathway for TGF-β. TGF-β binding leads to the assembly of a hetero-tetrameric receptor complex in which the type II receptor phosphorylates and activates the type I receptor. Pathway-restricted SMADs (Smad2 and Smad3), which may be anchored in the cytoplasm in homotrimeric forms, are phosphorylated, which leads to heteromerization with Smad4, a common-mediator SMAD. The hetero-oligomeric complex is then translocated to the nucleus, where it binds directly or in complex with other component(s) to DNA and affects transcription of specific genes. Note that it is not known if the hetero-oligomer between Smad2, Smad3 and Smad4 is a hexamer or has another stoichiometry. **b**, Inhibitory SMADs (Smad6 and Smad7) bind to the receptors, and prevent the phosphorylation and signalling activity of pathway-restricted SMADs. Whether inhibitory SMADs occur as monomers or multimers is not known.

munication) and *C. elegans* (Sma-4). The presence of homologue of both pathway-restricted SMADs and common-mediator SMADs in lower species suggests that complex formation of these two types of SMAD molecules may be a conserved mechanism for signalling downstream of serine/threonine kinase receptors.

Functional roles of SMAD/MAD domains. Structural studies and studies of SMAD mutants have now provided an insight into the functional roles of the different domains in SMADs (summarized in Fig. 4).

The MH2 domain of SMADs may serve as an effector domain in signal transduction, as suggested by its ability to induce a transcriptional response when fused to a yeast GAL4 DNA-binding domain[34] and by the finding that the MH2 domain of Smad2 induces a full range of activin responses in the absence of the MH1 domain[35]. However, in Smad4 the MH2 domain alone is not sufficient for signal transduction: a part of the linker region (termed the Smad4 activation domain[52]) that is not conserved in other SMADs is required for signalling activity, together with the MH2 domain[50,52]. Moreover, the MH2 domains of Smad1, Smad2 and Smad3 mediate homomeric interactions and are responsible for activation-induced interactions with Smad4 (refs 50, 53, 54).

In the resting cell, SMADs are localized in the cytoplasm. Stimulation with ligand leads to translocation to the nucleus. The observation that mouse Smad2 containing the linker region and MH2 domain localizes to the nucleus in the absence of ligand stimulation[35] suggests that the MH1 domain of Smad2 anchors the molecule in the cytoplasm. An alternative possibility is that truncation or ligand-induced heteromerization of SMADs leads to exposure of nuclear targeting sequence(s) in the linker region or the MH2 domain[50].

The MH1 domain of Smad4, and possibly of other SMADs, plays a role as a negative regulator by interacting with the MH2 domain, thereby preventing hetero-oligomer formation between pathway-restricted SMADs and common-mediator Smad[54]. Phosphorylation of the C-terminal SSXS motif in pathway-restricted SMADs appears to remove this inhibition. Mutations in SMAD MH1 domains have been reported in certain cancers, at Arg 133 in Smad2 and at the corresponding Arg 100 in Smad4 (refs 36, 55); these mutant SMADs form homo-oligomers but cannot form Smad2–Smad4 hetero-oligomers and cannot transduce signals. This inhibition occurs by increased affinity of the mutated MH1 domains to the corresponding MH2 domains, which leads to an augmentation of the auto-inhibitory function of the MH1 domain[54].

In addition to its role as a repressor of the MH2 domain, the MH1 domain, together with part of the linker region, may also be involved in direct DNA binding as indicated by studies on *Drosophila* Mad[56]. In *Drosophila*, Mad mediates the Dpp-dependent transcription of the *vestigial* (*vg*) gene through a sequence-specific DNA-binding activity. Binding of Mad to the 'quadrant' enhancer of *vg* was observed, but only when the MH2 domain was removed[56]. Thus, for the binding of the MH1 domain of Mad to the *vg* quadrant enhancer, the MH2 domain appears to have a repressor function. It remains to be determined whether mammalian SMAD MH1 domains also have direct DNA-binding activity.

Three-dimensional structure of SMADs. The three-dimensional structure of the MH2 domain of Smad4 has been determined by crystallography at 2.5 Å resolution[57]. It consists of a β-sandwich with antiparallel β-sheets, capped at one end by a three-α-helix bundle and at the other by three large loops and an α-helix (termed the loop/helix region). The MH2 domain of Smad4 forms a homotrimer in the crystal with the loop/helix region of one subunit interacting with the three-α-helix bundle of another. Size estimates by gel chromatography support the notion that the MH2 domain, as well as the intact Smad4 molecule, occur as trimeric structures in solution as well[57]. The MH2-domain trimer is in the form of a disc, with the amino terminals of all monomers, where the MH1 domains would be attached, phasing the same side. The other side of the disc may interact with other SMAD homotrimers in the heteromeric complex. The mutations in MH2 domains detected in human cancer cells may disrupt the structure of the oligomer: some mutations disrupt the folding of the protein; others, located at the loop/helix region or the three-α-helix bundle, prevent the formation of the homotrimer; others, located at a loop (L3) in the loop/helix region which is exposed on the surface of the disc, disrupt the formation of heteromers but not of homotrimers, indicating that this region may be exposed on the surface of the disc and be critical for heteromer formation[57]. In all cases, the assembly of heteromeric complex is prevented and the cell is deprived of antiproliferative TGF-β signals.

Activation of SMADs. Maximum transcriptional effect requires the cooperation between pathway-restricted SMADs and Smad4 (refs 37, 38, 48). Activation of type I receptors triggers the assembly of heteromeric complexes of the two types of SMADs, by phosphorylation of pathway-restricted SMADs in their C-terminal SSXS motifs. The mechanism may involve a phosphorylation-induced unfolding of the N- and C-terminal domains, allowing interaction with Smad4 to occur, and/or a direct interaction between the phosphorylated tail of pathway-restricted SMADs and Smad4 (ref. 46). Given the trimeric structure of Smad4 (ref. 57), such complexes may be hexamers, but their exact stoichiometry is unknown. Observations suggesting that other configurations of the active complex are possible is that full activity in a transcriptional assay can only be achieved when Smad2, Smad3 and Smad4 are all present, and that not only does Smad4 interact with Smad2 and Smad3, but Smad2 and Smad3 also interact with each other in a TGF-β-dependent manner[38].

Inhibitory SMADs. Smad6 and Smad7 diverge structurally from other members of the SMAD family[58–61]: whereas they share sequence similarity with other SMADs in their C-terminal domains, their N-terminal regions (36% identical between Smad6 and Smad7) differ from those of other SMADs. Inhibitory Smads have also been detected in *Xenopus* (Smad8; J. Christian, personal communication) and *Drosophila* (Dad; ref. 62). Smad6 and Smad7 function as inhibitors of TGF-β, activin and BMP signalling. They bind to type I receptors and interfere with the phosphorylation of the pathway-restricted SMADs. Consequently, active heteromeric Smad complexes are not formed. A requirement for binding of inhibitory SMADs to type I receptors is the activation of type I

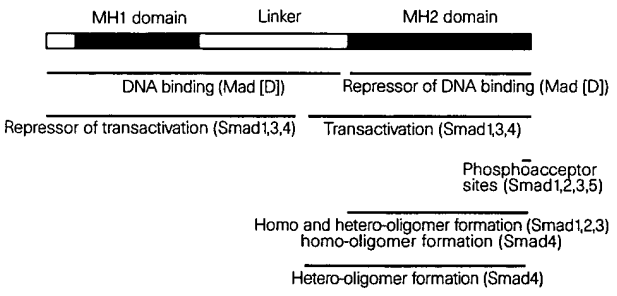

Figure 4 Different functional domains in SMADs. The conservation of MH1 and MH2 domains in the SMADs suggests that these domains may have similar functions in different members of the SMAD family, but it remains to be shown to what extent observations made on individual members (in parentheses) can be generalized.

receptor by type II receptor kinase. However, inhibitory SMADs show a more stable interaction with type I receptors than do pathway-restricted SMADs. As pathway-restricted SMADs can compete with inhibitory SMADs for binding, a plausible mechanism for inhibition is to prevent the receptor interaction and phosphorylation of pathway-restricted SMADs (Fig. 3b). In an analogous way, Dad blocks the *Drosophila* phenotype induced by activated receptor or Mad[62], suggesting that Dad may directly interfere with the function of Mad.

Transcription of inhibitory SMAD mRNA is induced by stimulation by TGF-β as well as by other stimuli (ref. 59; and M. Kawabata *et al.*, unpublished observation), and in *Drosophila* Dad is induced by Dpp[62]. Thus, inhibitory SMADs may act as autoregulatory negative-feedback signals in the signal transduction of the TGF-β superfamily.

Transcriptional regulation by SMADs

TGF-β family members mediate their multifunctional effects by eliciting transcriptional responses on many target genes. The responsive elements in the promoters of some of these genes have been mapped and interacting transcription factors identified. In most cases, however, it is still unclear whether these genes are direct targets, and it is unknown whether SMADs are directly involved in their transcriptional regulation. Activin induces the transcription of the homeobox gene *goosecoid*[63] and the forkhead/winged-helix transcription factor *XFKHI* (ref. 64) without requirement of protein synthesis. Putative SMAD target genes in the BMP pathway include *Xom*[65], *Xvent*-1 (ref. 66) and *Msx*-1 (ref. 67). TGF-β potently induces transcription of plasminogen activator inhibitor-1 (ref. 68) and itself[69], with involvement of the AP-1 transcription factor, as well as the cyclin-dependent-kinase (CDK) inhibitors p15 (ref. 70) and p21 (ref. 71), with involvement of Sp1. Whether SMADs interact with AP-1 and Sp1 is not known.

Studies on the activation of *Xenopus Mix.2* and *Drosophila vg* provide the strongest evidence so far for a function of SMADs as transcriptional modulators downstream of serine/threonine kinase receptors. The homeobox gene *Mix.2* is an early-response gene induced by TGF-β superfamily members during early *Xenopus* development[72]. Smad2, Smad4 and FAST-1, a new member of the winged-helix transcription factor family, are components of an activin-responsive factor (ARF) that interacts directly in an activin-dependent manner with a 6-base-pair repeat in the activin-response element of the *Mix.2* promoter[72]. FAST-1 is the principal DNA-binding component in ARF. A C-terminal domain (amino acids 380–506) of FAST-1, termed SMAD-interacting domain (SID), is involved in binding to Smad2 and Smad4, and overexpression of SID specifically inhibits activin signalling[73]. The C-terminal part (amino acids 453–506) of SID is essential for the association of FAST-1 with Smad2, which occurs in the absence of Smad4. Phosphorylation of Smad2 enhances the interaction between Smad2 and FAST-1. The N-terminal part (amino acids 380–453) of SID appears to be required for the interaction of Smad4 to the Smad2–FAST-1 complex, but Smad4 does not directly bind FAST-1 in the absence of Smad2. Binding of Smad4 may stabilize the Smad2–FAST-1 complex as an active DNA-binding complex. The activin-response elements in promoters of *goosecoid* from different species[63], and *Xenopus XFKHI/XFD-1* (ref. 64) have been mapped and show little sequence similarity with the activin-response element in the *Mix.2* gene. This suggests that different SMAD-containing transcription factor complexes can be formed which show different DNA-binding specificities.

Drosophila Mad was shown to bind to a (G + C)-rich sequence and to be essential for activation of quadrant enhancer of *vg*[56]. The binding of Mad to DNA appears to be of low affinity, although it is specific, suggesting the need for heteromeric complex formation with a Smad4 homologue and/or other cofactors for high-affinity binding. This is also indicated by the finding that overexpression of the C-terminal domain of SMADs is sufficient to mimic the effects of ligand stimulation[35] and suggests that the principal DNA-binding component in the transcription factor complex is not provided by a SMAD. An intrinsic low-affinity DNA-binding activity in the MH1 domain may be complemented by specific interactions with other transcription factors through the MH2 domain.

Genetic analysis of Dpp signalling in *Drosophila* has implicated Schnurri (Shn) as an essential downstream component of Dpp-dependent signalling in embryonic endoderm pattern formation[74,75]. The *Shn* gene encodes a putative zinc-finger transcription factor with similarity to mammalian transcription factors of the major histocompatibility complex (MHC)-binding protein family. Shn activity, rather than its expression, appears to be regulated by Dpp. Thus, it is possible that Mad interacts directly with Shn to regulate transcriptional responses.

TGF-β family members may act as morphogens and induce concentration-dependent responses. These responses can be reproduced with increasing doses of SMADs[35,76], so it is possible that the level of nuclear SMAD provides a direct readout for the level of ligand-induced receptor activation. Promoters with different affinities for SMAD-containing transcription factor complexes may thus become activated in cells along a concentration gradient of TGF-β family members.

Receptor-interacting proteins

SMADs are clearly crucial for signal transduction of members of the TGF-β family. Using yeast two-hybrid screens, other molecules interacting with type I and type II serine/threonine kinase receptors have also been identified, which may modulate receptor signalling.

The FK506-binding immunophilin FKBP12 interacts with unstimulated TβR-I and other type I receptors. FKBP12 is not a substrate for the receptor kinase, and TβR-I mutants that are unable to interact with FKBP12 can still signal positively[77–79]. FKBP12 binds to a Leu-Pro sequence in the GS domain of type I receptors[77,79] (Fig. 1), and counteracts phosphorylation of the type I receptors by type II receptors; it is released from the type I receptors after ligand-induced receptor activation[78]. Thus, FKBP12 protects against ligand-independent, spontaneous activation of type I receptors by type II receptors[79].

Also, the α-subunit of farnesyltransferase can interact with TβR-I[80–82]. TGF-β stimulation does not alter the farnesyltransferase activity in mink lung cells, however, and this enzymatic activity is dispensable for the antiproliferative effect on TGF-β and its transcriptional responses in these cells[81]. Using a similar methodology, molecules that interact with TβR-II have also been identified—namely apolipoprotein J (ref. 83) and a WD-domain-containing protein, TRIP-1 (ref. 84), whose functional importance is not known.

Other cytoplasmic signalling pathways

In addition to the pathways already described, other parallel pathways may exist that could be important for the transduction of specific signals. Examples include TAK-1, a serine/threonine kinase of the MAP kinase kinase kinase family, which is activated by TGF-β or BMP-4 (ref. 85), and members of the Ras[86] or Rac[87] families of small GTP-binding proteins which also have been implicated in TGF-β signalling. Certain MAP kinases, such as the extracellular signal-regulated kinases (ERK)1 and 2 and stress-activated protein kinase (SAPK)/Jun-N-terminal kinase (JNK), have also been reported to be activated by TGF-β in certain cell types[88,89]. Several of these pathways are efficiently activated in response to other signalling molecules. This may thus be yet another example of crosstalk between different signalling pathways, which appears to be common in signal transduction.

Subversion of signalling in tumorigenesis

TGF-β has a multifunctional role in tumorigenesis. At early stages,

when cells still respond to its antimitogenic effect, TGF-β may act as a tumour suppressor. However, during malignant progression, when cells acquire an insensitivity to growth inhibition by TGF-β, it may function as a tumour promoter by stimulation of angiogenesis, immunosuppression and synthesis of extracellular matrix, which provides an appropriate microenvironment for rapid tumour growth and metastasis. The biphasic action of TGF-β in tumorigenesis was demonstrated in a mouse skin model of multistage carcinogenesis using transgenic mice with keratinocyte-targeted TGF-β1 expression[90].

The escape from the antimitogenic response of cells by TGF-β during tumour progression suggests a potential function for components in the TGF-β signal transduction pathway as tumour suppressors[91]. Support for a tumour-suppressor role for the type II receptor of TGF-β came from the analysis of an inherited form of colon cancer with a microsatellite instability phenotype[92]. Moreover, the frequent mutation and homozygous deletion of *Smad4* in pancreatic cancers led to its original discovery as the *DPC4* tumour-suppressor gene[93] (Fig. 2b). Loss of Smad4 expression has been identified in various TGF-β-resistant cancer cells, and transfection of Smad4 in these cells rescues responsiveness to TGF-β[37,52,55]. The MH2 domain of Smad4 is often the target for point mutations and frameshift mutations that lead to premature stops. Mutations in the MH2 domain may disrupt the core structure of the protein, or perturb the ability to form stable homotrimers or hetero-oligomers with pathway-restricted SMADs, depending on which amino-acid residues are mutated[57] (see above). Somatic mutations in *Smad4* are frequently observed in pancreatic cancers[93], but less frequently in other types of cancers such as colon, breast and lung cancers. Functionally disruptive mutations in *Smad2*, a gene that is located close to Smad4 on chromosome 18, have so far been noted only in colorectal and lung cancers[36,94] (Fig. 2). *Smad1*, *Smad3*, *Smad5* and the MH2 domain of *Smad6* (*JV15-1*) do not appear to be frequently mutated in colon, breast, lung and pancreatic cancers[95]. The finding of a higher frequency of somatic mutations in *Smad4* than in other *Smad* genes is consistent with a unique and non-redundant role for the common partner Smad4 in TGF-β superfamily signalling.

TGF-β induces growth inhibition by upregulation of the CDK inhibitor p15 in certain epithelial cell lines[96]. However, analysis of p15-defective human cancer cell lines reveals that the antiproliferative effect of TGF-β is also mediated by repression of the expression of Cdc25A, a CDK tyrosine phosphatase which activates CDK[97]. Cdc25A is transcriptionally induced by c-Myc[98], and c-*myc* expression is repressed by TGF-β with similar kinetics to Cdc25A (refs 97, 99); an interesting possibility is therefore that c-Myc is involved in the transcriptional regulation of Cdc25A by TGF-β.

Conclusions

In view of their crucial importance in embryonal development, it is no surprise that signalling by TGF-β members is carefully regulated. Several intracellular control mechanisms have been discussed here. In addition, there are observations indicating that signalling is also regulated at several extracellular steps, including activation from latent precursor complexes, and interactions with specific binding proteins and accessory receptors.

Remarkable progress has been made in unravelling a signalling pathway for TGF-β and related molecules from the cell membrane all the way to the nucleus (Fig. 3). SMADs are key components in these signal transduction pathways. At present, nine vertebrate SMADs are known (Fig. 2a), but the family probably contains several other members. After phosphorylation and activation by receptor kinases, hetero-oligomeric SMAD complexes migrate into the nucleus and, either directly or in complex with other proteins, affect transcription of specific genes. Thus, the overall mechanism is reminiscent of that of signal transducers and activators of transcription (STAT) molecules, which, after phosphorylation by cytokine-receptor-associated JAK tyrosine kinases or tyrosine kinase receptors, dimerize and move into the nucleus and induce the transcription of specific genes[100].

An important finding is that certain SMADs serve as inhibitors in the signal transduction of members of the TGF-β superfamily by preventing the interaction between the serine/threonine kinase receptors and pathway-restricted SMADs. As expression of the inhibitory SMADs is induced by ligand stimulation, they may have a negative-feedback role in signal transduction. Likewise, feedback switch-off signals have been found in several other signal transduction pathways and emerge as a common theme in signal transduction.

Although we are gaining our first insight into the three-dimensional structure of SMAD[57] and the involvement of SMADs in direct[56] and indirect[73] binding to promoter regions in specific genes, many important issues remain unresolved. Is the basis for specificity of interaction between type I receptors and different SMADs due to specific docking epitopes or to the substrate specificities of the receptor kinases? Is receptor-induced phosphorylation in the C-terminal tail sufficient for SMAD activation and initiation of all subsequent downstream signalling events, or are other phosphorylation events necessary? By which mechanism do SMADs translocate from the cytoplasm to the nucleus? Which are the partners of SMADs in the transcriptionally active complexes, and which are the target genes? Given the level of activity in the field, answers are likely to come along soon.

Note added in proof: Tyrosine kinase receptor-mediated activation of MAP kinase was recently shown to lead to phosphorylation of Smad1 in the linker region and inhibition of its translocation to the nucleus[101]. Cross-talk between different types of signalling pathways may thus occur by differential regulation of Smad1 activation. □

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